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Human Reproduction, Vol.24, No.9 pp. 2244–2254, 2009
Advanced Access publication on June 2, 2009 doi:10.1093/humrep/dep200

human reproduction

ORIGINAL ARTICLE *Reproductive biology*

Effect of fetal or neonatal exposure to monobutyl phthalate (MBP) on testicular development and function in the marmoset

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BACKGROUND: Fetal exposure of male rats to some phthalates induces reproductive abnormalities, raising concerns for similar effects in humans. In order to address this in a more appropriate animal model, the aim of the present studies was to investigate the effect of fetal/neonatal exposure to monobutyl phthalate (MBP) in a non-human primate, the marmoset. In particular, to determine if exposure resulted in effects at birth, or in adulthood, similar to those in male rats, and whether there was evidence for induction of carcinoma-in-situ (*CIS*) or testicular germ cell tumours (TGCT).

METHODS: Pregnant female marmosets were dosed from ~7–15 weeks gestation with 500 mg/kg/day MBP and male offspring studied at birth (1–5 days; $n = 6$) or in adulthood ($n = 5$). In another study, newborn males ($n = 5$ co-twins) were dosed with 500 mg/kg/day MBP for 14 days, commencing at ~4 days of age.

RESULTS: Fetal exposure of marmosets to MBP did not affect gross testicular morphology, reproductive tract development or testosterone levels at birth, nor were germ cell number and proliferation, Sertoli cell number or germ:Sertoli cell ratio affected. In two of six MBP-exposed animals, unusual clusters of undifferentiated germ cells were found, but their significance is unclear. Neonatal MBP treatment did not affect germ cell numbers or differentiation. Fetal exposure to MBP did not affect testis size/morphology, germ cell numbers or fertility in adulthood. There was no evidence for *CIS* or TGCT.

CONCLUSIONS: Fetal exposure of marmosets to MBP does not measurably affect testis development/function or cause testicular dysgenesis, and no effects emerge by adulthood. Some effects on germ cell development were found, but these were inconsistent and of uncertain significance.

Key words: fetal / germ cells / monobutyl phthalate / neonatal / testis

Introduction

Reproductive disorders in newborn boys (cryptorchidism, hypospadias) and young men [low sperm counts, testicular germ cell tumours (TGCT)] are common and/or increasing in incidence (Skakkebaek *et al.*, 2001). These disorders are risk factors for each other and may comprise a testicular dysgenesis syndrome (TDS) with a common origin in fetal life (Skakkebaek *et al.*, 2001; Sharpe and Skakkebaek, 2008). Abnormal testis development leading to altered somatic cell (Sertoli and/or Leydig cell) function, such as altered testosterone production by the fetal testis during the period of the male programming window (Welsh *et al.*, 2008), may underlie TDS disorders (Sharpe and Skakkebaek, 2008).

Evidence suggests that environmental factors/exposures can affect the risk of TDS (Sharpe and Skakkebaek, 2003). Several studies have shown that fetal exposure of male rats to a common environmental chemical, di(*n*-butyl) phthalate (DBP), or to certain other phthalates, can induce a spectrum of disorders in male offspring similar to TDS (Mylchreest *et al.*, 2000; Parks *et al.*, 2000; Fisher

et al., 2003). Human exposure to phthalates such as DBP is ubiquitous (Silva *et al*., 2004; CDC, 2005), and exposure during pregnancy (Marsee *et al*., 2006) results in fetal exposure (Silva *et al*., 2004), raising concerns that this could contribute causally to TDS. In rats, fetal testosterone production is inhibited by *in utero* exposure to DBP (Lehmann *et al*., 2004; Thompson *et al*., 2004) or its main metabolite monobutyl phthalate (MBP) (Shono *et al*., 2000). Similarly, exposure to either DBP (Mylchreest *et al*., 1998; Fisher *et al*., 2003) or MBP (Shono and Suita, 2003; Shono *et al*., 2005) results in a high incidence of cryptorchidism, while MBP exposure in rats also results in reduced fertility in some male offspring (Kai *et al*., 2005).

While there is indirect evidence to suggest that certain phthalates could impair fetal testosterone production in humans (Swan *et al*., 2005; Swan, 2008), data from *in vitro* (Hallmark *et al*., 2007; Lambrot *et al*., 2008) and *in vivo* (Huang *et al*., 2009) studies suggest the opposite. Furthermore, fetal testosterone production in mice is unaffected by DBP, MBP or monoethylhexyl phthalate (MEHP) (Gaido *et al*., 2007). In contrast, phthalate effects on germ cell numbers and/or differentiation in the fetal testis have been shown in mice (Gaido *et al*., 2007), rats (Ferrara *et al*., 2006) and *in vitro* in the human (Lambrot *et al*., 2008). This raises concerns, as impaired fetal germ cell development underlies the origins of TGCT in humans (Rajpert-de Meyts, 2006).

Rodents are not good models for human fetal germ cell development (Mitchell *et al*., 2008) and, in view of the contrasting effects of phthalates on fetal steroidogenesis in rats and mice described earlier, it is unclear whether the rat is a suitable model for the human. We have therefore used a non-human primate, the marmoset, to investigate if phthalate exposure perinatally can affect testis development. The marmoset is similar to the human in terms of developmental periods in the male (Kelnar *et al*., 2002), perinatal germ cell differentiation (Mitchell *et al*., 2008) and organisation and (low) efficiency of spermatogenesis (Millar *et al*., 2000; Sharpe *et al*., 2000). It may therefore be a better model for the human than rodents. We have already shown (Hallmark *et al*., 2007) that neonatal administration of MBP can (transiently) inhibit testosterone production similar to the fetal effects of DBP in rats (Thompson *et al*., 2004). However, there are no reports of testicular effects of fetal exposure to MBP or other phthalates in the marmoset.

The present studies had two aims. First, to determine if TDS-like effects, similar to those found in DBP-exposed rats (Fisher *et al*., 2003), could be induced in marmosets following fetal or neonatal exposure to MBP. Second, to determine if fetal MBP exposure resulted in adverse testicular effects in adulthood, in particular whether there was evidence of impaired spermatogenesis/fertility or induction of TGCT or its precursor, *CIS*. Our results show no major, consistent effect of MBP exposure on testis and reproductive development, although some (inconsistent) effects on germ cell development need further investigation.

Materials and Methods

Animals, treatments, sample collection and processing

Animals were captive-bred common marmoset monkeys (*Callithrix jacchus*), maintained in a closed self-sustaining colony since 1973. Two studies were undertaken. In the first, nine pregnant females were administered 500 mg/kg/day MBP (TCI Europe, Zwijndrecht, Belgium) from ~7–15 weeks of gestation. Regular, systematic palpation and/or ultrasound were used to diagnose pregnancy and estimate gestational age. Experience has shown this to be accurate to within one week and was subsequently confirmed by time of birth. A total of 11 male offspring originated from these treated mothers and were killed either around birth (1–5 days; $n = 6$) or in adulthood ($n = 5$). The treatment time window was chosen based on comparative analysis of fetal testis development in the marmoset and human using cell-specific markers (Mitchell *et al*., 2008 and unpublished data) and which indicated that this period will encompass seminiferous cord formation and onset of testosterone production (marmosets have a disproportionately long embryonic period). This period is thought to correspond to a time window in the rat which is critical for androgen-dependent programming of reproductive tract masculinisation (Welsh *et al*., 2008). Animal welfare considerations precluded the use and treatment of large numbers of animals, so to ensure reasonable numbers of MBP-treated animals, we restricted the number of control newborn males (born to vehicle-gavaged pregnant mothers) to $n = 3$ and supplemented these with 7 untreated controls to ensure adequate numbers of controls. These supplementary controls were 1–5 day-old animals which had been killed because they originated from triplet births, in which only two animals usually survive. For all parameters investigated in the present studies, values for the supplementary controls were comparable to those for the three offspring from vehicle-treated mothers. For evaluation of testes in adulthood, males exposed *in utero* to MBP were compared with control animals ($n = 5$) of comparable age that were derived from other experiments (e.g. Lunn *et al*., 1994, 1997), but whose mothers had not been treated during pregnancy.

In the second study, 10 newborn marmosets were used, comprising five pairs of co-twin males (one vehicle-treated, one MBP-treated), which enabled pair-wise evaluation using smaller numbers of animals than would otherwise be necessary (Sharpe *et al*., 2002). Commencing at 4 days of age, marmosets were orally administered vehicle or 500 mg/kg/day MBP for 14 days. The MBP was dissolved in dimethyl sulfoxide then suspended in honey and was taken voluntarily by infants (Hallmark *et al*., 2007). MBP is considered the active metabolite of DBP and can induce the same fetal testicular effects as DBP in rats (Shono *et al*., 2000, 2005) and its administration to newborn marmosets (at the presently used dose) has been shown to significantly reduce testosterone levels in blood (Hallmark *et al*., 2007). MBP was used in the present studies because marmosets are reported to be poor metabolizers of orally administered phthalate diesters to their monoesters (Rhodes *et al*., 1983, 1986). This means that once the maximum metabolic conversion capacity of a pregnant female marmoset has been reached after DBP dosing, administration of higher doses will not result in any further metabolism, and therefore exposure, to the monoester (MBP). Therefore, to ensure maximum possible exposure in the present studies we administered MBP not DBP. We chose to administer 500 mg/kg/day as this dose of either DBP (Fisher *et al*., 2003; Mahood *et al*., 2007) or MBP (Shono *et al*., 2000, 2005) causes major adverse effects on the fetal testis in rats and (MBP) can suppress testosterone levels in newborn marmosets (Hallmark *et al*., 2007).

Animals were killed at 1–5 days or 18–21 months of age (fetal exposure) or 4 h after the last (neonatal) treatment, via intraperitoneal injection of an overdose of sodium pentobarbitone (Euthatal; Rhone Merieux Ltd, Harlow, UK). Testes were dissected out, fixed for 6 h in Bouin's fixative, weighed, then transferred to 70% ethanol and processed into paraffin wax in a Leica automatic processor (Leica Microsystems, Milton Keynes, UK).

For all studies, animals were treated humanely and with regard for alleviation of suffering. Studies were performed according to the Animal (Scientific Procedures) Act 1986 under UK Home Office Project Licence approval, and also approved by the local ethical committee for studies in primates.

Table I Antibodies and conditions used for immunohistochemistry

Antigen	Source	Species	Dilution	Retrieval	Target
3β-HSD[a]	Gift	Rabbit	1:800	N	Leydig cells
SMA[b]	Sigma	Mouse	1:1000	N	Peritubular myoid cells
Cytokeratin	Santa Cruz	Mouse	1:500	Y	Sertoli cells
AMH[c]	Santa Cruz	Goat	1:1000	N	Sertoli cells
OCT4	Santa Cruz	Goat	1:40	Y	Undifferentiated gonocytes
C-KIT	Dako	Rabbit	1:20	Y	Undifferentiated gonocytes
AP-2γ	Santa Cruz	Mouse	1:40	Y	Undifferentiated gonocytes
VASA	Abcam	Rabbit	1:200	Y	Germ cells
N-Cadherin	Zymed	Mouse	1:200	Y	Cell adhesion molecule
SOX9	Chemicon	Rabbit	1:100	Y	Sertoli cells
Ki67	Dako	Mouse	1:40	Y	Proliferating cells

[a]3b-Hydroxysteroid dehydrogenase;[b]smooth muscle actin; [c]anti-Müllerian hormone.

Plasma levels of testosterone

Plasma testosterone was measured using an ELISA adapted from an earlier radioimmunoassay, as described previously (Fisher *et al*., 2003). The limit of detection was 12 pg/ml.

Immunohistochemistry

Specific proteins were detected by immunohistochemistry using methods detailed previously (Fisher *et al*., 2003; Gaskell *et al*., 2004; Ferrara *et al*., 2006; Hutchison *et al*., 2008; Mitchell *et al*., 2008). In the case of N-Cadherin and SOX9, protocols were optimised using a range of antibody dilutions and appropriate positive and negative control tissues. The antibodies, dilutions and requirement for antigen retrieval are shown in Table I. Briefly, antigen retrieval used 0.01 M citrate buffer, pH 6.0, except for SOX9, which required retrieval in 0.5 M glycine and 0.01% (w/v) EDTA, pH 3.5. Endogenous peroxidase was blocked by incubating slides in 3% (v/v) H_2O_2 in methanol and endogenous biotin was blocked using an avidin/biotin blocking kit (Vector Laboratories Inc., Peterborough, UK) according to manufacturer's instructions. After incubation overnight at 4°C with primary antibody, slides were washed in 0.05 M Tris-buffered saline (TBS), pH 7.4, then incubated with appropriate biotinylated secondary antibody, followed by incubation with streptavidin-conjugated horseradish peroxidase (Dako, Ely, UK) and visualisation of immunostaining using diaminobenzidine (Liquid DAB+, Dako).

Double immunostaining using Fast Blue visualisation

VASA/OCT4: after incubation with the primary (VASA) and secondary antibodies as described earlier, slides were washed in TBS, incubated with streptavidin-conjugated alkaline phosphatase (Dako) diluted 1:200 in TBS, and immunostaining visualised using 1 mg/ml Fast Blue (Sigma-Aldrich Ltd, Poole, UK) until staining was optimal. After washing in TBS, slides were immunostained for OCT4 using DAB visualisation as described earlier.

Ki67/VASA: immunostaining for Ki67, using DAB detection, was performed as described earlier. Slides were then washed in TBS and immunostained for VASA using Fast Blue as earlier.

To ensure reproducibility of results and accurate comparison of immunostaining between treatment groups, sections from all groups were run in parallel on at least two occasions. Negative controls were included in each experiment, for which the primary antibody was replaced with either peptide-preabsorbed antibody (OCT4) or the appropriate normal serum (all other antibodies), and in all such cases immunostaining was absent. Representative sections were photographed using a Provis AX70 microscope (Olympus Optical, London, UK) fitted with a Canon DS6031 digital camera (Canon Europe, Amsterdam, The Netherlands). Images were compiled using Photoshop CS2 (Adobe Systems Inc., Mountain View, CA, USA).

Determination of germ cell and Sertoli cell numbers

Sections were analysed using a Zeiss Axio-Imager microscope (Carl Zeiss Ltd, Welwyn Garden City, UK) fitted with a Hitachi HV-C20 camera (Hitachi Denshi Europe, Leeds, UK) and a Prior automatic stage (Prior Scientific Instruments Ltd, Cambridge, UK). Image-Pro 6.2 with Stereologer plug-in software (MagWorldwide, Wokingham, UK) was used to select random fields for counting and to place a grid over the tissue. Germ cell counting used sections double-immunostained for VASA and OCT4 (1–5 days of age) to label all subpopulations, VASA alone (17–20 days of age) or sections stained with hematoxylin (adults); in adults, spermatogonia, spermatocytes and round spermatids were counted separately, while elongate spermatids and spermatozoa were combined. Sertoli cell counting used sections immunostained for SOX9, a Sertoli cell-specific nuclear marker (Swain and Lovell-Badge, 1997). Relative cell volume per testis was first determined (Sharpe *et al*., 2003). The number of fields counted per animal (~15–75 fields) was dependent on obtaining a percentage standard error value of <5%. Data were converted to absolute volume per testis by multiplying by testis weight (equivalent to volume), then converted to cell number per testis after determination of mean cell nuclear diameter and volume (average of 70–100 nuclei) using the Stereologer software nucleator function. For elongate spermatids and spermatozoa, only cell volume per testis was calculated due to extreme variation in shape of the nucleus.

Determination of the germ cell PI

PI was determined stereologically on sections double-immunostained for Ki67/VASA, which enabled identification of both proliferating and non-proliferating germ cells. A total of ~150 germ cells, from two sections per animal, were counted. The proliferation index (PI) was calculated as

Table II Expected number of MBP-exposed marmosets exhibiting testicular or reproductive tract abnormalities, based on the incidence induced in rat studies using the same dose of the parent compound DBP[a]

Abnormality	Incidence in rat studies[b]	Number of affected marmosets[c]	
		Expected	Observed
Hypospadias	17%	2	0
Cryptorchidism	≥70%	≥7	0
Small testes/impaired spermatogenesis	≥70%	≥7	0
Focal testicular dysgenesis	≥50%	≥5	0

[a]As MBP is considered to be the active moiety, exposure of the marmosets to MBP should have been at least equivalent (and probably more) to that in rats administered the same dose of DBP; [b]Incidence observed in previous studies (Fisher *et al.*, 2003; Mahood *et al.*, 2007); [c]Expected number based on 11 MBP-exposed offspring studied at birth or in adulthood and assuming the same % incidence as found in our rat studies.

the number of $Ki67^+/VASA^+$ cells divided by the number of $Ki67^+/VASA^+$ plus $Ki67^-/VASA^+$ cells × 100.

Statistical analysis

All data were analysed using the Student's *t*-test (two-tailed). Paired *t*-test was used for the co-twin study.

Results

Effect of *in utero* exposure to MBP on normality of reproductive tract development

Our findings are summarised in Table II. We compared these with the incidence of abnormalities observed in our studies in rats (Fisher *et al.*, 2003: Mahood *et al.*, 2007), and calculated the number of animals expected to exhibit each abnormality based on a total of $n = 11$ MBP-exposed male offspring studied and assuming the same percentage incidence as observed in the rat studies. All males exposed *in utero* to MBP were normally masculinised at birth and showed no evidence of hypospadias; this was confirmed when males were killed either at age 1–5 days (termed 'at birth', $n = 6$) or in adulthood (18–21 months; $n = 5$). At birth, testes could be visualised in transit through the inguinal canal and were similarly placed in MBP-exposed animals and controls. Plasma testosterone levels in MBP-exposed animals at birth (2.32 ± 0.76 ng/ml; mean ± SEM; $n = 6$) were comparable to controls (2.41 ± 0.72 ng/ml; $n = 5$). When killed, no gross abnormalities of the epididymis, vas deferens, prostate or seminal vesicles were apparent in MBP-exposed males or in controls (data not shown).

Effect of *in utero* exposure to MBP on testis morphology and size at birth

Immunohistochemistry for cell-specific markers was used to evaluate normality of testis formation/morphology; immunostaining for smooth muscle actin (SMA) (Fig. 1A and B) was used to label peritubular myoid cells, cytokeratin (Fig. 1C and D) and anti-Müllerian hormone (AMH) (Fig. 1E and F) for Sertoli cells and 3β-hydroxysteroid dehydrogenase (3β-HSD) for Leydig cells (not shown). Immunostaining for these markers was comparable in control and MBP-exposed animals at birth and seminiferous cord formation, size and number and general testis morphology appeared normal (Fig. 1). Leydig (3β-HSD-immunopositive) cells were relatively few in number at this age but were located normally in the interstitium in MBP-exposed animals and controls. Examination of several sections from each animal revealed no malformed cords or foci of dysgenesis (Table II). Testis weight was also comparable in the two groups (Table III). Only one unusual morphological feature was found in two MBP-exposed animals. On testis sections immunostained for AMH, large unstained areas within seminiferous cords were noted which were occupied by germ cell clusters (Fig. 2A), so these were investigated in more detail.

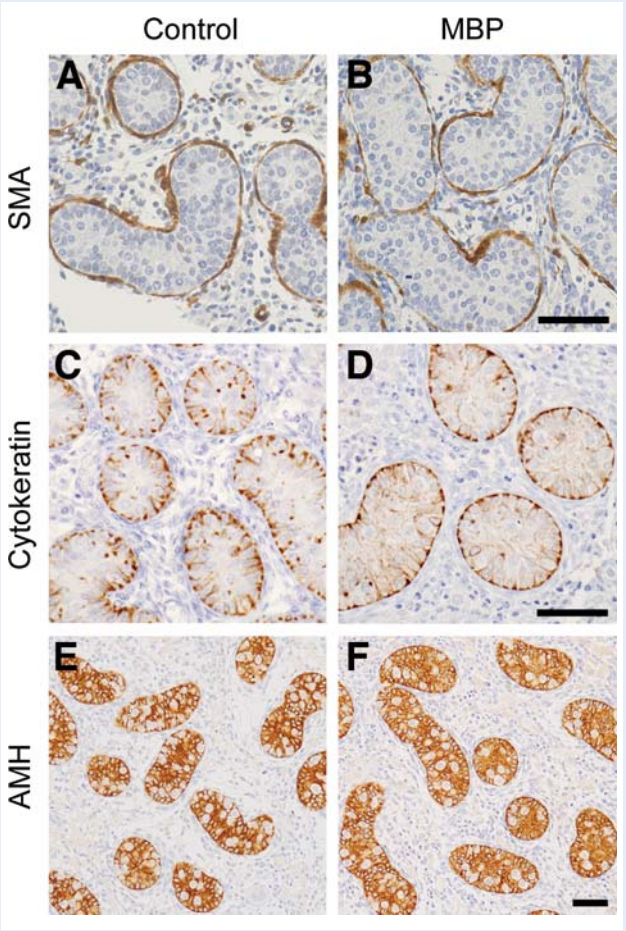

Figure 1 Representative testicular histology and immunoexpression of smooth muscle actin (SMA) (A and B), cytokeratin (C and D) and anti-Müllerian hormone (AMH) (E and F) in 1–5-day-old marmosets after fetal exposure to 500 mg/kg/day MBP or in controls. Immunoexpression of all three proteins in MBP-exposed animals was comparable to controls. Seminiferous cord structure and size appeared normal, with no evidence of focal dysgenesis. Sertoli cell differentiation and function was similarly unaffected. Bar = 50 μm.

Table III Testis and body weights (means ± SEM) in the marmosets used for the present studies, and in other (unquantified) adults for comparison

Treatment	Age at evaluation	No. of animals	Testis wt (mg)	Body wt (g)
Controls (vehicle-treated)	1–5 days	3	5.5 ± 0.8	29.7 ± 1.0
Controls (non-treated)		7	4.7 ± 0.4	29.0 ± 1.9
Controls (combined)		10	4.9 ± 0.6	29.2 ± 1.3
MBP 500 mg/kg/day		6	4.8 ± 0.6	29.3 ± 1.9
Controls (vehicle-treated)[a]	17–20 days	5	11.5 ± 0.9	56.8 ± 4.1
MBP 500 mg/kg/day[a]		5	11.0 ± 0.8	51.8 ± 3.8
Controls (quantified)[b]	Adult	5	522 ± 85	366 ± 15
Controls (not quantified)[c]		12	516 ± 9	388 ± 17
Controls (combined)		17	518 ± 24	381 ± 13
MBP 500 mg/kg/day		5	605 ± 34	411 ± 21

[a]Co-twin males; [b]Untreated control adults most closely age-matched to MBP-exposed animals; [c]Data from other untreated adults, showing that quantified adults are representative.

Germ cell aggregation/clusters at birth in MBP-exposed animals

Immunostaining of serial testis sections with AMH (Fig. 2A), OCT4 (Fig. 2B), AP2γ and C-KIT (not shown) confirmed the presence of unusual clusters of germ cells in the two aforementioned MBP-exposed animals. Almost all of the germ cells in these clusters were immunopositive for OCT4 (Fig. 2B), AP2γ and C-KIT (not shown), indicating that they were undifferentiated gonocytes. In one animal, several clusters were observed in every section examined from different regions of both testes; in the second animal, clusters were less frequent but distributed in different parts of the testis. Germ cell clusters were also observed in sections from 2 of 10 control animals, but were much fewer in number, sporadic and much smaller in size. In the two MBP-exposed animals, the clusters typically contained 10–20 germ cells, but clusters of up to 27 germ cells were found. In controls, clusters rarely contained more than 10 germ cells.

Abnormal germ cell aggregation occurs in the centre of seminiferous cords in fetal testes of DBP-exposed rats, due to withdrawal of Sertoli cell cytoplasm from around the germ cells (Kleymenova *et al.*, 2005). We investigated this using immunochemistry for the adhesion protein N-Cadherin (Johnson and Boekelheide, 2002). This showed N-Cadherin immunoexpression in MBP-exposed animals with germ cell clusters comparable to controls (Fig. 2C), suggesting that cell–cell adhesion in clusters was normal. Immunostaining of serial sections with OCT4 and Ki67 revealed no clusters of $Ki67^+$ cells corresponding to $OCT4^+$ cell clusters (data not shown), ruling out the possibility that clusters resulted from unusual germ cell proliferation. The etiology of the germ cell clusters therefore remains unexplained. Multinucleated gonocytes are induced by DBP treatment of rats (Kleymenova *et al.*, 2005), but none were found in MBP-exposed marmosets or controls.

Effect of *in utero* exposure to MBP on germ cell numbers and differentiation at birth

The neonatal marmoset testis contains a mixed complement of germ cells, some expressing the pluripotency marker OCT4, others expressing the differentiation marker VASA, and a small population expressing both (Mitchell *et al.*, 2008). This enables distinction of the least differentiated gonocytes ($OCT4^+/VASA^-$) from the most differentiated spermatogonia ($OCT4^-/VASA^+$) with some germ cells intermediate ($OCT4^+/VASA^+$). Double immunostaining for OCT4 and VASA was therefore used to identify all germ cells for counting and to discriminate the relative proportions of undifferentiated versus differentiated germ cells.

Total germ cell number per testis varied considerably between animals, ranging from 0.12 to 0.56 $\times 10^6$ in controls and from 0.29 to 0.84 $\times 10^6$ in MBP-exposed animals. The majority of germ cells in both groups were $VASA^+$ (Fig. 3A), while $OCT4^+/VASA^+$ cells comprised only 1–2% of the total (not shown). There was no significant difference in numbers of $VASA^+$ cells, $OCT4^+$ cells or in total germ cell number per testis in MBP-exposed animals compared with controls (Fig. 3A).

Germ cell differentiation, quantified by expressing $OCT4^+$ germ cells as a percentage of the total germ cell population, varied widely (0.4–28.9%) between control animals. There was no significant change in MBP-exposed animals, although in one animal, the percentage of $OCT4^+$ cells was far above the control range (5× the mean control value) (Fig. 3B).

Double immunohistochemistry for Ki67 and VASA was used to determine the PI in $VASA^+$ germ cells. Because $OCT4^+$ germ cells comprised on average only 12% of the total at this age, and because of the difficulty in labelling their nuclei for both Ki67 and OCT4, we did not determine PI in $OCT4^+$ cells. In testes from control animals, the mean PI of $VASA^+$ cells was 28% (Fig. 4), with marked variation between animals. There was no significant effect of MBP exposure on $VASA^+$ germ cell proliferation (Fig. 4), although in one MBP-exposed animal the PI was unexpectedly high and more than double the mean control value. This was not one of the animals described earlier in which we found extensive germ cell clustering.

Effect of *in utero* exposure to MBP on Sertoli cell number and the germ cell:Sertoli cell ratio at birth

Sertoli cell number in controls was ~4 $\times 10^6$ per testis and was unaffected by MBP exposure (Fig. 5A). There was a non-significant trend

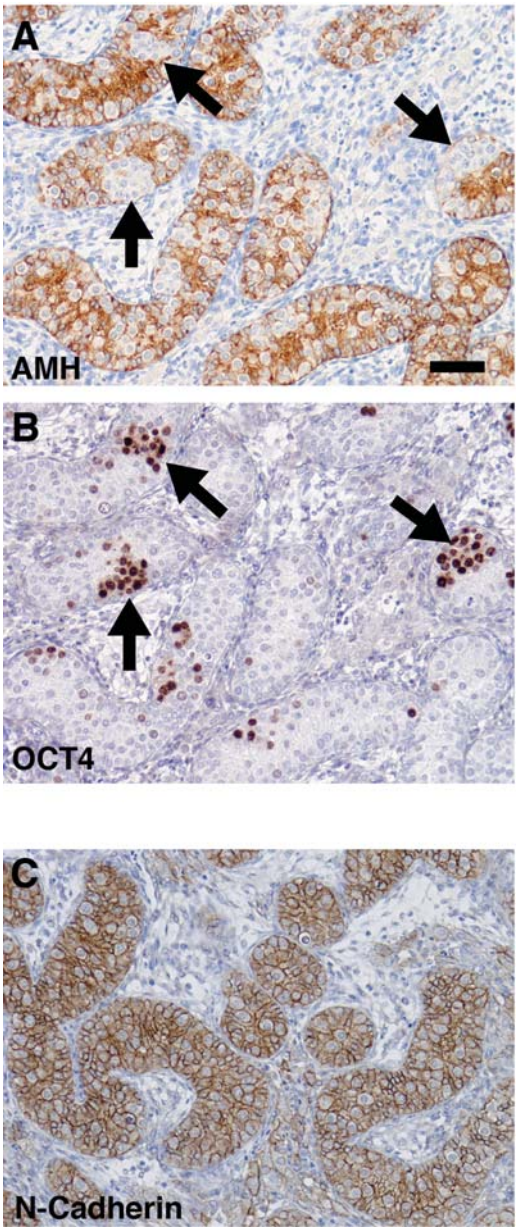

Figure 2 Appearance of germ cell clusters observed in a 1–5-day-old marmoset after fetal exposure to 500 mg/kg/day MBP. Serial testicular sections were immunostained for anti-Müllerian hormone (AMH) (A) and OCT4 (B). Arrows indicate AMH^- areas within seminiferous cords corresponding to clusters of $OCT4^+$ germ cells. N-Cadherin immunoexpression (C) in the same MBP-exposed animal was comparable to controls and immunonegative areas were absent. Bar = 50 μm.

towards an increase in the germ cell:Sertoli cell ratio in MBP-exposed animals (Fig. 5B). However, in one MBP-exposed animal, the ratio was well outside the control range (~3× the mean control value). This animal had the lowest number of Sertoli cells in the MBP-exposed group and the highest number of germ cells and was the animal (described earlier) in which the most extensive clustering of germ cells was found.

Effect of neonatal treatment with MBP on germ cell numbers and differentiation

Because there was ~12 weeks between cessation of fetal treatment and evaluation (around birth), during which MBP effects might disappear, we also studied male co-twin marmosets treated with vehicle or 500 mg/kg/day MBP for ~14 days. Testis weights in MBP-treated co-twins were comparable to controls (Table III). In control co-twins, OCT4 expression fell dramatically compared with animals at birth, with $OCT4^+$ cells either absent or occurring only sporadically in control testes. MBP-treated co-twins were comparable to controls (not shown), suggesting no effect on germ cell differentiation neonatally. Total germ cell number was determined by quantifying $VASA^+$ cells, and in controls this gave a mean (±SEM; $n = 5$) value of $1.63 \pm 0.20 \times 10^6$ (Fig. 6), indicating a 2.5-fold increase in germ cell number compared with values at birth (Fig. 3A). Germ cell number was reduced by 45 and 35% in two out of five co-twins exposed neonatally to MBP, when compared with their control co-twins, but was increased by 25% in another MBP-exposed co-twin (Fig. 6). Overall, there was no significant difference between control and MBP-treated co-twins (Fig. 6). There was no straightforward relationship between testis weight and germ cell number in individual animals.

Effect of *in utero* exposure to MBP on the adult marmoset testis and fertility

To determine if *in utero* exposure to MBP had any long-term effects on the marmoset testis or on fertility, some animals were evaluated in adulthood by comparing them with (control) animals previously used in other experiments. All testes were scrotal in MBP-exposed animals (Table II) and testicular morphology was completely normal (Fig. 7); testis weights were also comparable to controls (Table III). Examination of sections from different parts of both testes from each animal revealed no abnormal seminiferous tubules or foci of dysgenesis (Table II). Quantification of germ cells confirmed normal spermatogenesis in MBP-exposed animals (Table II), with no effect on numbers of different germ cell types (Fig. 8). Immunohistochemistry for OCT4 was used to look for presence of fetal-like germ cells, but none were detected in testes from control or MBP-exposed animals. Fertility was assessed in three MBP-exposed animals and was normal, but was not investigated in others.

Discussion

The aim of this study was to determine if fetal or neonatal exposure of male marmosets to MBP resulted in effects on testicular (focal dysgenesis, effects on Sertoli and germ cells) and reproductive tract (epididymal or penile abnormalities, testis maldescent) development similar to those found in rats exposed to phthalates, when studied either at birth or in adulthood. As male marmosets exhibit similar phases of testis development (McKinnell *et al.*, 2001; Kelnar *et al.*, 2002) and germ cell differentiation (Mitchell *et al.*, 2008) to the human, whereas rodents show pronounced differences, the marmoset may be a better model than rodents for study of MBP effects. In particular, differences in germ cell differentiation between humans and marmosets on the one hand and rodents on the other hand, suggest that marmosets could be susceptible to induction of *CIS* (the precursor of TGCT in humans) unlike the rat (Mitchell *et al.*, 2008). Therefore,

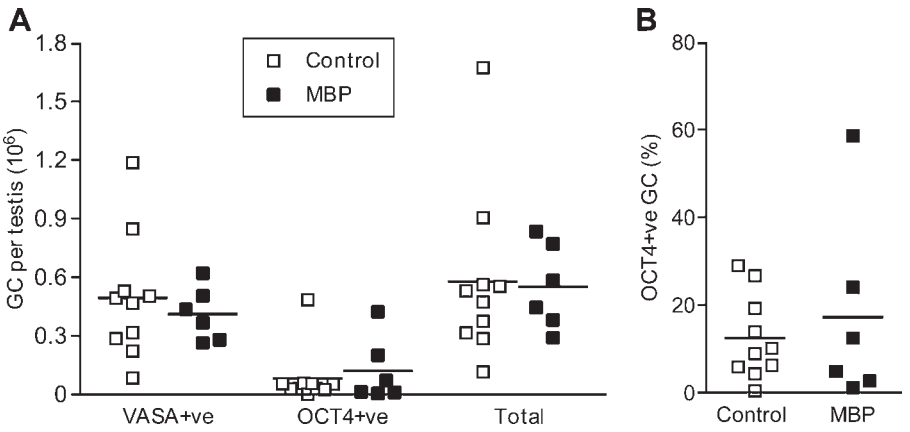

Figure 3 Germ cell (GC) numbers (A) and $OCT4^+$ cells as a percentage of total germ cell number (B) (a measure of germ cell differentiation) in testes from 1–5-day-old marmosets after fetal exposure to 500 mg/kg/day MBP or in controls.
Values shown are for individuals (horizontal line = mean); controls $n = 10$, MBP $n = 6$.

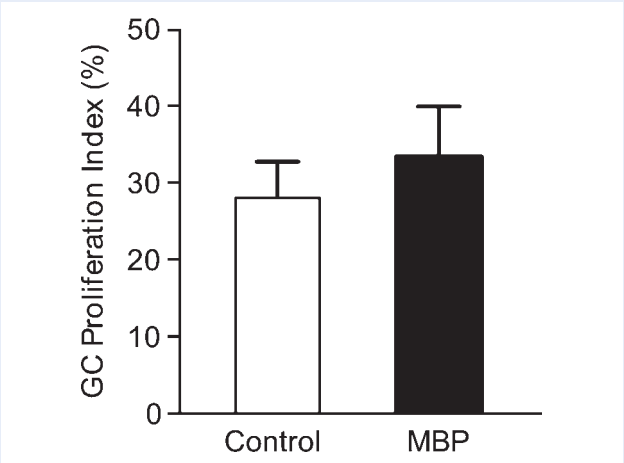

Figure 4 Germ cell (GC) proliferation in testes from 1–5-day-old marmosets after fetal exposure to 500 mg/kg/day MBP or in controls.
Values shown are means ± SEM; controls $n = 10$, MBP $n = 6$.

one focus of our studies was on perinatal germ cell differentiation. Treatment of pregnant marmosets for 7 weeks with a high dose of MBP did not induce any of the testicular or reproductive tract abnormalities in male offspring seen in similar experiments in rats (after DBP treatment), although there was limited, and inconclusive, evidence for effects on germ cells perinatally. No *CIS* or abnormalities in germ cell numbers or fertility were found in adult marmosets exposed fetally to MBP.

Several studies have shown that administration of DBP, or certain other phthalates, to pregnant rats induces TDS-like effects in male offspring (Parks *et al.*, 2000; Mylchreest *et al.*, 2000; Fisher *et al.*, 2003). Defects found neonatally include focal areas of seminiferous tubule dysgenesis (Fisher *et al.*, 2003), abnormal Leydig cell aggregates (Mahood *et al.*, 2005), occurrence of multinucleated gonocytes and abnormal gonocyte aggregation (Kleymenova *et al.*, 2005) and reduced germ cell numbers and delayed gonocyte differentiation (Ferrara *et al.*, 2006). Using similar immunohistochemical techniques to these studies in rats, we found no evidence for similar effects in MBP-exposed marmosets at birth or in adulthood.

One of the major effects of DBP/MBP exposure in rats is inhibition of testosterone production by the fetal testis (Shono *et al.*, 2000; Lehmann *et al.*, 2004) which leads to downstream effects such as reduced anogenital distance, cryptorchidism and hypospadias in occasional animals (Foster *et al.*, 2001; Fisher *et al.*, 2003) and reduced Sertoli cell number at birth (Scott *et al.*, 2007, 2008). We found no evidence for penile abnormalities or cryptorchidism in MBP-exposed marmosets either at birth or in adulthood, and Sertoli cell number was comparable to controls at birth. Although these findings do not rule out the possibility that MBP exposure reduced testosterone production by the fetal testis during treatment, they show that if this occurred, it was not sufficient to affect penile development. Based on rat studies, the critical phase for penile effects due to androgen suppression is during the male programming window, estimated to be within the MBP treatment period (~7–15 weeks gestation) when extrapolated from rat and human (Welsh *et al.*, 2008) and based on fetal testis development in the marmoset (Mitchell *et al.*, 2008).

A key focus of the present study was the potential effects of MBP exposure on germ cell development. Two main aspects were investigated, germ cell number and differentiation. We investigated the effect of fetal and neonatal MBP exposure in separate studies, as with fetal exposure there was ~12 weeks between cessation of treatment and evaluation (around birth), in which effects of MBP might have been recovered from, whereas this was not the case in neonatally-treated animals. Previous studies *in vivo* in rats (Ferrara *et al.*, 2006) and *in vitro* in rats and humans (Lambrot *et al.*, 2008) have shown that certain phthalates (DBP/MBP, MEHP) can reduce fetal germ cell number. We found that neither fetal nor neonatal exposure to MBP had any significant effect on germ cell numbers. However, in the neonatally treated animals, two of the five MBP-treated co-twins showed a marked reduction in germ cell number compared with their control co-twins. If this was due to MBP treatment, it is unclear why the remaining three MBP-exposed animals did not show evidence for a similar effect, so the difference could be unrelated to treatment.

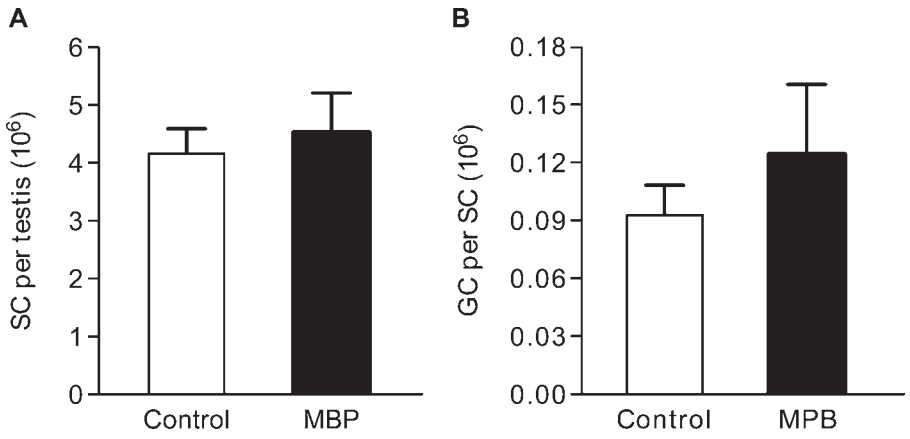

Figure 5 Sertoli cell (SC) number (A) and germ cell (GC):SC ratio (B) in testes from 1–5-day-old marmosets after fetal exposure to 500 mg/kg/day MBP or in controls.
Values shown are means ± SEM; controls $n = 6$, MBP $n = 6$.

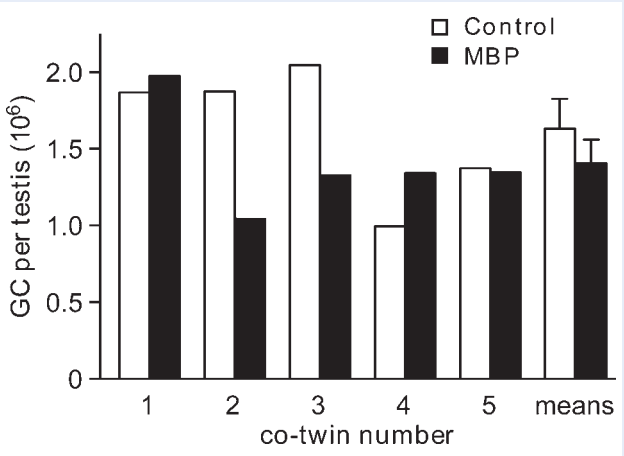

Figure 6 Germ cell (GC) number in testes of 17–20-day-old marmosets co-twins treated for the previous 14 days with either vehicle (control twin) or 500 mg MBP/kg/day.

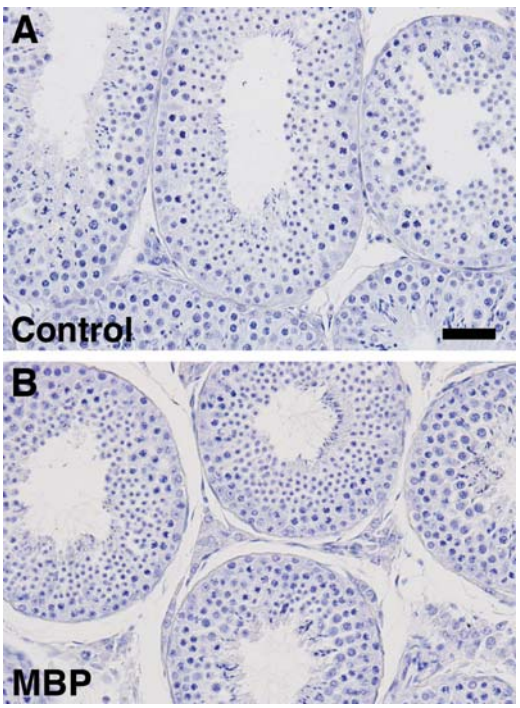

Figure 7 Representative testicular histology in adult marmosets after fetal exposure to 500 mg/kg/day MBP (B) or in controls (A). In MBP-exposed animals, seminiferous tubule structure and composition of seminiferous epithelium appeared comparable to controls. Bar = 50 μm.

In terms of relevance to the human, the most important aspect of germ cell development that we evaluated is their differentiation, because incomplete fetal germ cell differentiation is thought to underlie formation of *CIS* from which TGCT develops in adulthood (Rajpert-de Meyts, 2006). A previous study in rats showed that fetal DBP exposure delays (but does not prevent) germ cell differentiation (Ferrara *et al*., 2006). The marmoset appears an excellent model for the human as in both species perinatal germ cell differentiation is asynchronous (in contrast to the rat), and the testes at birth contain a mixed population of differentiated ($VASA^+$) and undifferentiated ($OCT4^+$) germ cells (Mitchell *et al*., 2008). In marmosets (Mitchell *et al*., 2008) and humans (Gaskell *et al*., 2004), the proportion of $OCT4^+$ germ cells declines progressively during fetal and neonatal life, as gonocytes differentiate. In the present study, in newborn control marmosets $OCT4^+$ cells comprised ~12% and $VASA^+$ cells ~87% of total germ cells. Although these proportions varied considerably between animals, in control animals ($n = 10$) $OCT4^+$ cells never comprised >29% of the total. If differentiation was delayed by MBP exposure, the proportion of $OCT4^+$ cells would be increased, but we found no evidence for this in either fetally or neonatally MBP-exposed animals. However, in one marmoset exposed fetally to MBP, the % $OCT4^+$ cells was outside the control range (~5-fold

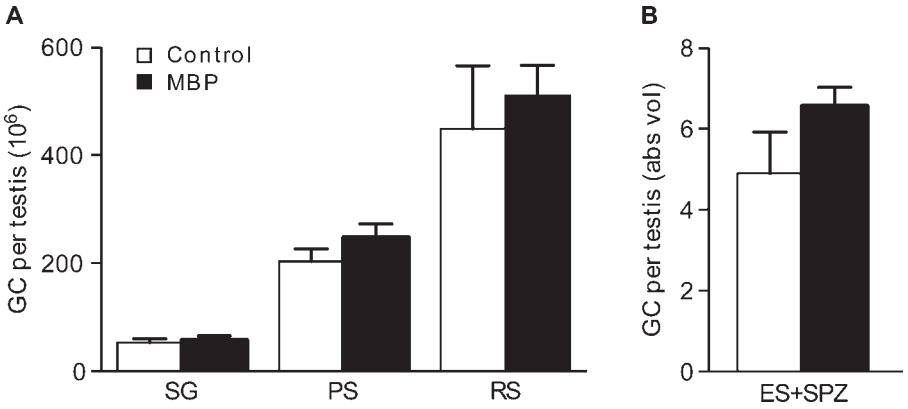

Figure 8 Numbers (A) of spermatogonia (SG), spermatocytes (PS) and round spermatids (RS), and absolute volume (abs vol) (B) of elongating spermatids plus spermatozoa (ES + SPZ) in testes of adult marmosets after fetal exposure to 500 mg/kg/day MBP or in controls. MBP had no significant effect on numbers of any germ cell type.

higher than the mean control value). This could indicate that OCT4 expression had been abnormally prolonged in some germ cells in this animal, but we cannot exclude the possibility that it was simply at the extreme of the normal range (based on 10 controls in this time period).

Another unusual finding in two marmosets after fetal MBP exposure, was the occurrence of large germ cell clusters. These mainly comprised cells immunopositive for OCT4, AP2γ and C-KIT, and therefore classified as undifferentiated gonocytes. Neither of these animals was the one referred to above with an unusually high proportion of $OCT4^+$ germ cells. Aggregation of germ cells has previously been observed in the testes of fetal rats exposed to DBP, due to withdrawal of Sertoli cell cytoplasm from around the germ cells (Kleymenova *et al.*, 2005). In the present study, however, germ cell clusters were more localised within the cords and generally bordered the basal lamina. Additionally, N-cadherin immunoexpression in Sertoli cell cytoplasm around the clusters indicated normal Sertoli cell-germ cell contact, unlike in the rat lesions. Exactly how these germ cell clusters arose is unclear, and we found no previous reference to such clusters in the literature. One possibility is that they are foci of clonal expansion, although no unusual pattern of Ki67 expression was observed within these areas.

Likewise it is not possible to conclude whether germ cells within these clusters are abnormal. Clusters contained a much higher proportion of $OCT4^+$ cells than in the germ cells outside of the clusters, perhaps suggesting that differentiation in these areas is occurring more slowly than in the testes as a whole. If the clusters represent foci of delayed development, it could be significant in relation to origins of *CIS* in humans (Rajpert-de Meyts, 2006), which is characterised by expression of the pluripotency marker OCT4 and other markers of undifferentiated or fetal germ cells such as AP2γ and C-KIT, which we also found in these germ cell clusters. However, since such clusters were found in 2 out of 10 control animals, albeit smaller in size and with much lower frequency, it is possible that the clusters in MBP-exposed animals are merely at the extreme end of the normal range. For certain, we found no such clusters or *CIS*-like cells in adulthood, but as this is based on only five animals exposed *in utero* to MBP, animal numbers may have been too low to detect this phenomenon or its consequences (if any). In view of its importance, this needs clarifying.

Based on the present studies, we conclude that administration of high doses of MBP to pregnant marmosets does not affect steroidogenesis by the fetal testis during the critical period of the male programming window, sufficient to cause any detectable downstream effects; nor is there any evidence for focal or wider testicular dysgenesis. This conclusion is based on a sufficient number of animals ($n = 11$ offspring in total) to provide a measure of confidence. While these are relatively small numbers, they should be considered in the context of the high incidence of abnormalities found in rat studies using DBP. Assuming that the high dose of MBP used in our study resulted in a similar incidence, we would have expected to see several animals exhibiting one or more abnormality.

However, this conclusion rests on the assumption that all effects of DBP are mediated via MBP in the marmoset, and we cannot exclude the possibility that the lack of effects of MBP in the present study is due to differences in metabolism of MBP by pregnant/fetal marmosets as compared with rodents.

While the findings also do not demonstrate any overt abnormality in germ cell numbers or development in MBP-exposed animals, some changes were detected at birth in a minority of animals that could indicate an effect, especially as only six animals were evaluated at birth for these parameters; this requires clarification. This is particularly the case in view of recent evidence indicating that MEHP has effects on germ cells but not on steroidogenesis during exposure of the human fetal testis *in vitro* (Lambrot *et al.*, 2008).

Funding

These studies were funded entirely by the UK Medical Research Council (WBS U.1276.00.003.00003.01).

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Submitted on March 5, 2009; resubmitted on April 27, 2009; accepted on May 6, 2009